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(71)(72) Applicant and Inventor: ROYER, Catherine, A. [US/US]; 424 Hillington Way, Madison, WI 53705 (US).

(74) Agents: SVENSSON, Leonard, R. et al.; Birch, Stewart, Kolasch & Birch, 8110 Gatehouse Road, Suite 500 East, P.O. Box 747, Falls Church, VA 22040-0747 (US).

(54) Title: QUANTITATIVE DETECTION OF MACROMOLECULES WITH FLUORESCENT OLIGONUCLEOTIDES

(57) Abstract

A method is described by which the association between an oligonucleotide labeled by attachment of a fluorophore and another macromolecule such as a protein or nucleic acid may be determined quantitatively in solution accuretely and with high sensitivity. In the performance of this method the polarization of fluoroescence of an extrinsic fluorescence probe that is covalently coupled to the oligonucleotide is determined. Changes in fluorescence polarization are related directly to the degree of association between the labeled oligonucleotide and another macromolecule and may be used to quantify the association. Because of its high sensitivity and accuracy, this method may be used to make reliable quantitative measurements of very small amounts of complexes formed between labeled oligonucleotides and proteins, nucleic acids or other macromolecules. The method also allows the accurate calculation of important biochemical parameters such as dissociation constants. The method, which is rapid and solution-based, has a broad range of application in biochemistry, genetic cloning and molecular biology, as well as in clinical diagnostics.

QUANTITATIVE DETECTION OF MACROMOLECULES
WITH FLUORESCENT OLIGONUCLEOTIDES

BACKGROUND OF THE INVENTION

Field of the Invention

5 The invention relates to the use of measurement of the polarization of the fluorescence emission from a labelled macromolecule in order to assess the binding of the labelled macromolecule to a second macromolecule. Thus, the invention is directed to analytic methods for
10 determining such binding in a quantitative fashion. The invention is also directed to apparatus for conducting binding analyses using measurement of fluorescence polarization.

Description of the Related Art

15 The use of labeled oligonucleotides as probes in macromolecular analysis is an important technique in molecular biology. Oligonucleotides have been labeled with radioisotopes, enzymes or fluorescent molecules. Because of the relatively low molecular weights of
20 oligonucleotides, and the common availability of instrumentation for their automated synthesis, oligonucleotides are often used in blot-hybridization procedures or in gel-retardation assays for the detection and qualitative evaluation of macromolecules
25 with which they may associate. These macromolecules may

2

be either proteins, RNA molecules or DNA molecules.

In a standard blot-hybridization procedure, the target macromolecule is separated by electrophoresis in a gel matrix, commonly agarose or polyacrylamide. It is

5   then transferred to a membrane in such a way as to preserve its relative spatial positioning within the gel matrix and fix it stably to the membrane. Alternatively, the macromolecule may be attached to the membrane without prior electrophoresis. The presence of

10 the macromolecule on the membrane is determined by binding to it a labeled oligonucleotide and subjecting the complex to autoradiography or, if the oligonucleotide is labeled with radioisotope, by scintillation counting.

15   In a standard gel retardation assay an oligonucleotide that has been labelled with radioisotope or other detectable moiety is electrophoresed in a gel matrix, commonly made of agarose or acrylamide, under non-denaturing conditions. The labelled oligonucleotide

20 is also associated with a protein that may bind to the oligonucleotide and the mixture is electrophoresed on a gel, commonly in a neighboring lane, for comparing with the unassociated oligonucleotide. Because of its higher molecular weight and less negative charge, the protein

25 will exhibit lower mobility in the gel than the unassociated oligonucleotide. If the oligonucleotide forms a stable complex with the protein, it will also exhibit a lower mobility than that of the unassociated oligonucleotide. Comparison of the mobility of the

30 oligonucleotide mobility in the presence and absence of the protein allows qualitative determination of whether a complex forms between the two macromolecules. These basic methods are used for a very large variety of determinations in basic genetic research, genetic

35 engineering, the medical sciences, and agricultural testing.

The present invention relates to a method for

detecting and quantitating complexation between nucleic acids and proteins or other macromolecules which comprises the measurement of the polarization of fluorescence of an extrinsic fluorophore covalently coupled to an oligonucleotide. The oligonucleotide can be an oligodeoxyribonucleotide, an oligonribonucleotide or a co-polymer of both. The nucleotide bases can be derivatized, as can the backbone chain. The oligonucleotide can be single-, double- or triple-stranded. The length of the oligonucleotide is determined by the specific experiment being conducted, but is preferably less than 40 residues long, more preferably being less than 25 residues long and most preferably 8-10 residues long.

The fluorophore is incorporated into the oligonucleotide at any position using standard automated DNA synthesis techniques and fluorescently labeled amino-linker compounds (e.g. those available from Clontech, Inc., La Jolla, CA). Alternately, unlabeled amino-linker compounds can be incorporated and subsequently labeled with the fluorescent compound. A variety of fluorophores may be used, including fluorescein, eosin, coumarin and dansyl derivatives. The fast rotation about the short axis of the oligonucleotide results in a low value of the fluorescence polarization of the probe covalently coupled to the oligonucleotide. This value is obtained by exciting the fluorescently labeled oligonucleotide with the appropriate wavelength of plane polarized exciting light and monitoring the emission of light polarized in the planes parallel and perpendicular to the plane of polarization of the exciting light. The fluorescence polarization is calculated as:

$$p = (I_{\parallel} - I_L)/(I_{\parallel} + I_L)$$

where $I_{\parallel}$ is the intensity of the emitted light polarized in a plane parallel to the plane of polarization of the

4

exciting light and I_L is the intensity of the emitted
light polarized in a plane perpendicular to the plane of
polarization of the exciting light.
The anisotropy of the emission is

5               $$2/3 [1/(1/p-1/3)]$$

This value is analogous to the polarization yet linear
with respect to the total fluorescence emission
intensity.

Fluorescence polarization is the basis for a series
10   of patented assays for the clinical detection of drugs,
steroids and other antigens (US Patents 4,269,511;
4,516,856; 4,585,862; 4,668,640; 4,784,961 4,902,630;
4,952,691; 5,066,426; European patent application
86102035.2). Although there are a number of variations,
15   all of these assays rely on the observation of a large
change in the fluorescence polarization of fluorescein
upon changing its interaction with specific or non-
specific antibodies when the drug of interest is present
in the assay solution. If the presence of the drug
20   results in the dissociation of the fluorescein-antibody
complex, for example, then the fluorescence polarization
will exhibit a large decrease. The sensitivity of these
assays is less than 10 pM.   Urios and Cittanova (15)
describe the use of fluorescently labeled Fab fragments
25   of antibodies to perform fluorescence polarization
measurements. The sensitivity of their assay is 2.5 μM.
The use of a fluorescent-labeled oligonucleotide probe
was reported by Murakami et al (17). Their reported
sensitivity was 100 nanomolar. Giedroc et al. (16) used
30   fluorescence anisotropy in evaluating the presence of
single stranded DNA molecules. Their assays were
conducted at oligonucleotide concentrations of 2 to 8
μM.

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SUMMARY OF THE INVENTION

One object of the present invention is to provide
a method by which the binding of two macromolecules to
each other can be detected in the solution phase by
5   measuring the polarization of the fluorescent emission
from a probe moiety. Preferably, the probe is attached
to one macromolecule and binding is determined directly
by an increase in the fluorescence polarization. A
second object of the present invention is to provide an
10   apparatus   suitable   for   performing   fluorescence
polarization measurements with very high sensitivity so
as   to   be   able   to   perform   such   measurements
quantitatively on very small samples. In preferred
embodiments   of   the   apparatus,   detection   of   the
15   polarization of the fluorescent emission of as little as
one femtomole of fluorophore can be detected.

A third object of the present invention is to
provide   diagnostic   methods   which   are   based   upon
measuring the binding of a polynucleotide to a second
20   polynucleotide, or alternatively to a protein, by means
of quantitation of the polarization of the emission from
a   fluorophore   covalently   attached   to   either   said
polynucleotide.

BRIEF DESCRIPTION OF THE DRAWINGS

25   **Figures 1A** shows the titration of a fluorescein-labeled
double-stranded oligodeoxynucleotide containing a trp
operator   sequence   with   purified   tryptophan   repressor
protein.   The buffer conditions were 10 mM phosphate,
0.1 mM EDTA, 10% glycerol, pH 7.6 in presence of 0.4 mM
30   L-tryptophan.   Total sample volume was 700 μl.   The
concentration of the fluorescein-labeled oligonucleotide
was 200 pM.   The anisotropy of fluorescence is plotted
as   a   function   of   the   logarithm   of   the   protein
concentration expressed as monomer.   The lines through
35   the points represent the non-linear analysis of the data
in terms of a model in which a monomer/dimer equilibrium

6

is coupled with the dimer-DNA binding equilibrium.

Figures 1C, 1D show the free energy diagrams for a
simple binding scheme and the protein-protein and
protein-DNA coupled interaction scheme. The data were
5   fit using these two models, as shown in Figures 1A and
1B.

Figure 2. Plot of X^2 (ordinate) vs. free energy in kcal
(abscissa) for the thermodynamic model given in Figures
1C.

10   **Figure 3** shows the dependence of the binding profile of
trp repressor to a synthetic oligonucleotide containing
a trp operator sequence on the concentration of the
oligonucleotide. The stoichiometry of the complexes
formed is a subtle function of the absolute and relative
15   concentrations of protein and oligonucleotide. Thus, a
primary plateau is reached which is more apparent at low
DNA concentrations, followed by a further increase in
the anisotropy as higher order complexes are formed.
DNA concentrations were 200 pM, 1 nM, and 20 nM.

20   **Figure 4** shows the dependence of the binding of the trp
repressor protein to the operator oligonucleotide upon
the concentration of tryptophan. Open circles represent
binding of protein to 1 nM oligonucleotide in presence
of 4.0 mM tryptophan, open triangles show binding in the
25   presence of 0.4 mM tryptophan, open diamonds 40 μM
tryptophan and the closed circles denote the binding
profile in absence of the tryptophan co-repressor.

Figure 5. Plan view drawing of a lamp-based, T-format
high sensitivity polarization fluorometer.

30   **Figure 6.** Plan view drawing of a laser-based, T-format
high sensitivity polarization fluorometer.

Figure 7. Plan view drawing of a lamp-based, L-format high sensitivity polarization fluorometer.

Figure 8. Plan view drawing of a laser-based, L-format high sensitivity polarization fluorometer.

5   Figure 9. Detail drawing in plan view of fiber optic, lamp-based, T-format high sensitivity polarization fluorometer, in particular showing detail of sample compartment and the arrangement of the film polarizers.

DETAILED DESCRIPTION OF THE INVENTION

10   The present invention defines methods by which the association between a fluorescently labeled oligonucleotide and another macromolecule such as a protein or a nucleic acid may be measured rapidly, accurately and with high sensitivity in solution. In
15   particular, this invention relates to the detection of complex formation (i.e., formation of a stable, specific noncovalent association) between the oligonucleotide and the target macromolecule, each at very low concentration, through the measurement of the
20   polarization of fluorescence of an extrinsic fluorescent probe covalently coupled to the oligonucleotide. The fluorescence polarization detection is based upon the increase in the rotational correlation time of the probe covalently linked to the oligonucleotide as a result of
25   the increase in the size of the tumbling particle when the protein or other macromolecule is complexed with it, compared to the rotational correlation time of the fluorescently labeled oligonucleotide in the absence of interacting macromolecules. Using the appropriate
30   instrumentation, measurements of fluorescence polarization may be made very rapidly, usually on the order or 5-10 minutes. In contrast, a blot-hybridization or gel retardation analysis, as described above, will take a full day -- and very often longer --

8

to complete.   Alternative uses of fluorescence
polarization measurements in macromolecular analysis
have been described (15-17). However, these methods do
not provide adequate sensitivity to make them useful in
5   practical applications. Biological macromolecules are
often found in extremely small amounts, requiring
analytical sensitivities at less than nanomolar levels.
The method described in the present invention allows
measurements to be made at the picomolar level -- well
10   within the range required and at least 10,000-fold more
sensitive than reported elsewhere. Alternative methods
of macromolecular analysis are largely qualitative and,
therefore, inaccurate.   The method of the present
invention provides for highly accurate quantitative
15   measurements.

High sensitivity, and thus a high signal-to-noise
ratio necessary to accurate quantitative analysis of low
concentrations of interacting molecules, is achieved
using an ILC 300 watt Xenon arc lamp/ISA monochromator
20   excitation source coupled to the sample compartment
through an Oriel rectangular to circular fused silica
fiber optic cable.   Film polarizers mounted on the
sample holder in a crossed T-format in emission and with
parallel orientation in excitation permit detection to
25   the 10 picomolar limit.   It is expected that since
fluorescence is proportional to the excitation source
intensity, the use of a laser excitation source will
result in a 10 to 100-fold increase in sensitivity, well
into the subpicomolar range of sample concentration.
30   Several other embodiments of the apparatus can be
used.   The T-format using fixed polarizing elements as
illustrated in Figures 5 and 6 can be changed to an L-
format using a rotatable emission polarizer. Use of a
laser illumination means eliminates the need for the
35   excitation polarizer element (as laser light is plane
polarized by its nature) and lenses for focusing the
excitation light. Use of a laser tunable to several

9

wavelengths, for instance a dye laser, provides for an apparatus having greater flexibility in the number of compounds which can be used as fluorescent labels in the assay of the present invention.

5   A preferred embodiment of the invention is an apparatus for measuring the polarization of fluorescent emission which can be understood by referring to the drawing Figures 5 and 6 and which comprises:

A box for the exclusion of light in which is placed
10   an illumination means (1) so as to illuminate a sample chamber (7), through a collimating lens (3) and an excitation focusing lens (4) for collecting the light and focussing it upon the sample chamber, and further through a first polarizing means for plane polarizing
15   the light from said illumination means (6), said lenses and first polarizing means, being mounted in a line connecting said illumination means and said sample chamber, and further said sample chamber being mounted between two additional polarizing means (8 and 8a), said
20   polarizing means being placed so that the line between them and including said sample chamber is perpendicular to the light path from said illumination means to said sample chamber, said polarizing means being mounted so that the plane of polarization of the second of said
25   polarizing means is parallel to the plane of polarization of said first polarizing means and also so that the plane of polarization of said second polarizing means is perpendicular to the plane of polarization of the third of said polarizing means and in which box is
30   also placed detection means (12 and 12a) for the collection and quantitation of the amount of light which passes through said polarizing means from said sample chamber to said detecting means. Interposed between the emission polarizers and detection means are emission
35   filters (9, 9a) for selectively passing light of a narrow range of wavelengths from the emission polarizers to the detecting means and also emission lenses (10,

10

10a) for focussing light emerging from the sample
chamber onto the detecting means. Shutters (11, 11a)
can also be interposed between the emission lenses and
detection means for controlling the entry of light into
5   the detection means and between the illumination means
and the sample compartment (11b in Figure 6) to control
entry of light into the sample compartment. Enclosed in
a separate compartment (13) are additional components of
the apparatus including motors for driving the shutters
10   and monochromator (if it is included), and electronic
devices for data collection and processing.

A second preferred embodiment of the invention is
an apparatus for measuring the polarization of
fluorescent emission which can be understood by
15   referring to drawing Figures 7 and 8 and which
comprises:

A box for the exclusion of light in which is placed
an illumination means (1) so as to illuminate a sample
chamber (6) through an excitation lens (3) for focussing
20   the excitation light upon the sample chamber and further
through an excitation filter (4), wherein said
excitation filter passes only a narrow range of
wavelengths of light, and finally through a fixed means
of plane polarizing light which shines on said sample
25   chamber (5), such that each of elements 3 and 4 and 5
are mounted in the light path from said illuminating
means to said sample chamber, said sample chamber being
mounted relative to a rotatable polarizing means (8),
which plane polarizes light which passes through said
30   rotatable plane polarizing means, so that a path from
said illumination means through said sample chamber to
said rotatable polarizing means forms a right angle with
the sample chamber at the vertex and in which box is
also placed an emission filter (7) interposed between
35   the sample chamber and said rotatable plane polarizing
means, wherein said emission filter will allow only a
narrow range of wavelengths of light to pass and in

which box is further mounted detection means (11) for
the collection and quantitation of the amount of light
which passes through said rotatable polarizing means
from said sample chamber to said detecting means.

5   Optionally interposed between the light source and the
excitation lens is a monochromator (2) for selected
particular wavelengths of light to be trained upon the
sample chamber. Interposed between said emission
polarizer and said detection means is an emission lens

10   (9) for focussing the light emerging from said sample
chamber upon said detection means and also an emission
shutter (10) for controlling the entry of light into
said detection means. Optionally interposed between
said illumination means and said sample compartment is

15   an excitation shutter (13 in Figure 8) for controlling
entry of light into the sample compartment. Other
components of the machine are separately contained (12),
including motors for rotating the emission polarizer,
and electronic devices and motors for controlling the

20   shutter and monochromator, and electronic devices for
data processing.

It can be easily understood by reference to figures
6 and 8 that when the light source is a laser, an
excitation polarizer is not required, as laser light is

25   intrinsically polarized. Similarly, the nature of laser
light makes the excitation collimating and focussing
lenses unneccessary.

The fluorescent label can be covalently bound to a
macromolecule by any of the many techniques commonly

30   known in the art. Oligonucleotide probes can be
labelled either along their backbone, at a position in
the organic bases, or at either end. End-labelling of
oligonucleotides with the fluorophore can be
accomplished by chemically coupling an N-

35   hydroxysuccinimide ester of the fluorophore to a 5'-
amino group of the oligonucleotide which is produced
during the synthesis of the oligonucleotide, or by using

12

a phosphoramidite reagent derivatized with the fluorophore in the synthesis of the oligonucleotide. An oligonucleotide can also be labelled at the 3' end by tagging it with fluorescently labelled nucleotides using

5   terminal transferase.

It is expected that the invention will be useful in the analysis of proteins, oligonucleotides, DNA, RNA and other macromolecules either biological or synthetic. Since the technique described in the present invention

10   is performed in the solution phase, it is particularly useful in identifying those parameters of the solution which promote or interfere with complex formation between polynucleotides and other macromolecules, for instance, pH, salt concentration or the presence of co-

15   factors. The sensitivity of the presently described technique is such that complex formation can be analyzed at concentrations of the macromolecules of less than 1 μM. The assay can easily be performed at macromolecule concentrations of 1 nM and can be done at concentrations

20   ranging from 10 to 100 pM.

Oligonucleotides labeled with a wide range of fluorophores, including but not limited to fluorescein, eosin and coumarin may be used as probes. The identity of the fluorophore does not define the invention. The

25   present invention encompasses any determination of the presence, character or amount of a macromolecule or any determination of specific intermolecular chemical parameters or constants in which measurement of the fluorescence polarization of a fluorescently labeled

30   oligonucleotide is employed.

The invention being thus described, preferred embodiments of the invention are set forth in the Examples below. These examples are meant only to be illustrative of the invention without being limiting of

35   its scope.

13

Example 1: Use of fluorescence anisotropy measurements to assay protein-nucleic acid interactions.

Fluorescence polarization of a fluorescently modified oligonucleotide has been used to study the complex

5   between an oligonucleotide and a protein. This was the case for the study of the interaction of Klenow fragment with a primer labeled with 5-dimethylamino-naphthalene-1-sulfonamide (12). However the goal of these studies was to gain specific structural

10   information about the complex. Due to the choice of the fluorophore and the instrumentation used in the study, the sensitivity of this assay was approximately 10 μM, 100,000 times less sensitive than the detection limit of the present invention. In fact, in those studies the

15   authors were unable to quantitate reliably the strength of the interaction between the protein and the oligonucleotide because the concentrations required for detection were at least 10,000 times greater than the value of the dissociation constant between the species.

20   The polarization of fluorescence of 7-dimethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin covalently bound to a 32 base pair double-stranded oligodeoxynucleotide containing the *lac* promoter sequence was used to monitor the formation of a complex

25   between the cAMP receptor protein and the lac promoter sequence (13). The concentration limit of the detection in this particular assay (10 nM) was several orders of magnitude above the interaction energy. It is also 1000-fold greater than the sensitivity of the assay

30   described in this invention, which can detect analytes at picomolar concentrations.

The methodology of the present invention is based upon the observation of changes in the anisotropy or polarization of fluorescence of a fluorescent probe

35   bound to a single or double-stranded oligonucleotide upon its interaction with a protein or other macromolecule. In the first set of experiments,

14

monitoring   protein-DNA   interactions,   the
oligodeoxyribonucleotide was a 25 mer which was labeled
on the 5'-end of the sense strand with fluorescein,
using a fluorescein-labeled phosphoramidite reagent in
5   automated DNA synthesis. The oligodeoxyribonucleotide
was obtained commercially. The sequence of the sense
strand was as follows (F = fluorescein):

       5'-F-CTTGCACTAGTTAACTAGTCAGCAA-3'

       The central CTAGAATTCTAG sequence is the specific
10   operator binding site of the tryptophan repressor
protein, which in the presence of its co-repressor
tryptophan, binds with high affinity (0.5 nM as
determined by gel retardation assays, Carey, 1988) to
the target sequence. Thus, the protein used in these
15   studies is the tryptophan repressor which was over-
expressed and purified using the method of Paluh and
Yanofsky (9), with modifications by Chou et al. (10).
       The anti-sense strand of the double-stranded
oligodeoxyribonucleotide was unlabeled. The two strands
20   were annealed by dissolution in 500 mM KCl, 10 mM
phosphate, 0.1 mM EDTA, pH 7.0 for final concentrations
near 10-100 μM. Equimolar mixtures of the two strands
as estimated from the optical density at 260 nm and the
sequence of the strands were prepared and were heated to
25   85°C for 10 minutes and allowed to cool slowly at room
temperature.
       The   fluorescein-labeled   double   stranded
oligodeoxyribonucleotide was   diluted to its final
concentration, usually 0.2 nM, in a total volume of 700
30   μl, directly into a reduced volume, 1.2 ml, fused silica
cuvette. The assay buffer used for the dilution, 10 mM
potassium phosphate, 0.1 mM EDTA, pH 7.6 also contained
10% glycerol and in some cases 0.4 mM L-tryptophan.
Upon dilution, the KCl concentration decreased to well
35   below 1 mM. The solution was allowed to equilibrate 5

15

minutes before each polarization measurement.

The fluorescence polarization or anisotropy was measured in L-format using an ISS KOALA™ automated fluorometer or in T-format using our original sample holder and polarizer arrangement for the ultrahigh sensitivity determinations. The exciting light was set at 488 nm and emission was monitored through a Y520 long pass filter from Hoya optics. The background fluorescence from the buffer, which is non-negligible at concentrations below 10 nM was subtracted by sequential measurement of the parallel and perpendicular components of the sample and the buffer in a matched cuvette.

Sensitivity and accuracy of the measurements.

The assay was performed by sequential addition of the protein in extremely small aliquots (2 μl) in order that the total concentration of the oligodeoxyribonucleotide not decrease by more than 10%, upon the change in volume. The plots in Figures 1A and 1B show that upon sequential addition of the protein to the solution containing the fluorescein-labeled oligodeoxyribonucleotide and 0.4 mM L-tryptophan, the anisotropy of the fluorescence increases in a sigmoidal fashion, when plotted against the logarithm of the protein concentration expressed as dimer. Each measurement was repeated 4 times and the results were averaged. The error on each measurement was +/- 0.002 polarization units. It can be seen that even when only 10% of the oligodeoxyribonucleotide, that is 20 pM, is bound by protein, the change in the anisotropy value is well beyond the error in the measurements.

We show in Figure 1A that subnanomolar quantities of oligodeoxyribonucleotide can be detected and its binding to protein produces a signal change which is 15-fold greater than the error of the measurement.

16

**Example 2: Use of fluorescence anisotropy measurements
to evaluat th rmodynamic models of protein-nucleic acid
interaction.**

These data were analyzed in terms of two
5   thermodynamic models, which are depicted in Figure 1C
and 1D. The first (1C) is a simple protein-DNA binding
model where the only species are the free protein, the
free DNA and the protein-DNA complex. In the second
model (Figure 1D), a monomer-dimer equilibrium is
10   superimposed upon the protein-DNA binding equilibrium.
The fits of the data using these two models and the
numerical equilibrium analysis program, BIOEQS, (20) are
shown in Figures 1A and 1B. The dissociation constant
for the tryptophan repressor dimer-DNA interaction as
15   obtained using the simple binding model was found to be
0.83 nM. The fit of the data using the linked
equilibria model yielded a dimer-DNA dissociation
constant of 0.23 nM and a dimer-monomer dissociation
constant of 3.5 nM. The quality of the data is
20   sufficient to rule out the simple binding model; the fit
is of much higher quality in the case of the linked
equilibria (c.f., Figure 1A and 1B).   Rigorous
confidence level testing reveals a steep X^2 well for the
dimer-DNA affinity, with 67% limits at +/- 0.3 kcal
25   (Figure 2). Thus, not only can complex formation at low
concentration be detected, but the quality of the data
is sufficient that the affinity can be determined from
the fit of the data, and the efficient evaluation of
alternative thermodynamic models can be made.

30   **Example 3: Use of fluorescence anisotropy measurements
in the analysis of multi-component systems in solution.**

The effect of changing the concentration of the DNA
oligonucleotide and of titrating the protein to the
concentrations found *in vivo* (near 1 μM) is shown in
35   Figure 3. It can be seen that complexes of higher
molecular weight and thus higher anisotropy are favored

17

at these higher concentrations of protein and DNA. Since this is a solution-based assay, the concentrations of any of the components can be varied easily. Thus, in Figure 4, one can see the effect of omitting the L-
5   tryptophan from the assay buffer.   Using 20 nM oligodeoxyribonucleotide, binding is only observed at protein concentrations above approximately 1 μM in repressor protein dimer in the absence of tryptophan co-repressor.

10   We have thus demonstrated that these assays can be used to test quantitatively the effects of specific ligands or other drugs on formation of complexes between proteins and nucleic acids.

The results shown in Figures 1 to 4 clearly
15   demonstrate that the polarization or anisotropy of fluorescence of fluorescently labeled oligodeoxyribonucleotides can be used to detect with high sensitivity and accuracy the interaction of the oligodeoxyribonucleotide with proteins and other large
20   biological molecules.   The fluorescence signal of fluorescein has been detected at concentrations as low as 10 pM in a total volume of 500 μl.   One femtomole of a particular DNA binding protein is easily detected, using 10 femtomoles of the fluorescently labeled target
25   sequence,   given   that   binding   of   10%   of   the oligonucleotide results in a signal change that can be measured accurately.   High sensitivity and data quality provide a means for the detection of complex formation and   also for the extremely accurate determination of
30   the energetic strength of the interaction, as well as the discrimination between thermodynamic binding models. The assay is quite rapid, taking only 5-10 minutes per point.   It is solution based, such that the effects of small ligands and drugs, pH, salt, temperature, and
35   other solution conditions can be evaluated easily.   For example, it would require approximately 10 minutes to ascertain whether crude cell extract or an in vitro

18

translation assay contained a protein factor which had
the ability to bind to a particular DNA sequence.   A
complete titration curve and its analysis requires
approximately 45 minutes. Its ease of use, solution-
5   based nature and rapidity make this assay a significant
improvement over methods based on electrophoresis or
filter-binding. This assay may be applied widely in the
study of transcription factors and in the role of drugs
and   other   small   molecules   in   regulating   genetic
10   expression. Additional applications include clinically
oriented tests for assaying function of transcription
factors or other DNA binding proteins.

**Example 4:   Use of Fluorescence anisotropy measurements
to assay interactions between nucleic acids.**
15   The binding of a single-stranded oligonucleotide to
another   complementary   single   stranded   DNA   or   RNA
oligonucleotide   can   also   be   detected   using   this
methodology.   Demonstration of significant changes in
anisotropy of fluorescence were made using   the 5'-
20   fluorescein   labeled   self-complementary   20 ·   mer
oligodeoxyribonucleotide,

5'-F-CGAACTAGTTAACTAGTACG - 3'

(F indicates fluorescein).   The change in anisotropy
25   occurs upon conversion of the denatured single-stranded
oligodeoxyribonucleotide to double-stranded form by the
formation of a double-stranded complex with itself.
Three tests were conducted.   In each, two samples were
compared. Each of the two samples contained labeled
30   oligodeoxyribonucleotide in 500 μl of 10 mM KPO_4 0.1 mM
EDTA, pH 7.6 at a concentration of 50 nM.   One of the
two samples (high salt) contained in addition 1M KCl.
The high salt sample was heated to 95°C and cooled to
room temperature. The heating and cooling at high salt
35   results   in   conversion   of   largely   single-stranded

19

oligodeoxyribonucleotide into double-stranded form.
This change in form should result in reduced Brownian
motion with a consequent increase in anisotropy.
Fluorescence anisotropy measurements were made on each
5   pair of samples. The results ·of these measurements,
given in Table I,   demonstrate an increase in
fluorescence anisotropy of at least 20-fold over the
error in the measurement upon formation of the double-
stranded complex.

10   Table I

                DETECTION OF OLIGONUCLEOTIDE HYBRIDIZATION
                             IN SOLUTION

Test Number	Anisotropy Low salt, before heating	Anisotropy High salt, after heating
1	0.046 +/- 0.001	0.067 +/- 0.000
2	0.042 +/- 0.001	0.065 +/- 0.000
3	0.044 +/- 0.001	0.066 +/- 0.001

        The change would have been much larger if the
20   oligonucleotide had been 8-10 bases long, or if the
     target sequence had been single-stranded plasmid DNA, as
     is also typically used in hybridization assays. Thus,
     the detection of the formation of the double-stranded 20
     mer is well below the size differential typically
25   involved when a small oligonucleotide binds to a large
     single-stranded DNA molecule.
        The assay based on measurement of nucleic acid
     interactions has application in genetic mapping and in
     the clinical determination of the existence of specific
30   mutations. It may also be used in the evaluation of
     translation inhibition by anti-sense DNA/RNA nucleic
     acids, a technique with potential applications in
     clinical diagnostics, therapeutics, or other biomedical
     areas.   The fluorescence anisotropy measurements

20

described herein are completely solution-based (i.e.,
require no solid support), a feature that will simplify
automation of the assay. In conclusion, this invention
represents a fast, accurate, reproducible, simple
5   quantitative assay for the interactions of nucleic acids
with other macromolecules.

**Example 6: Clinical assay for function of a DNA-binding
protein in a tissue biopsy**

The method of the present invention is expected to
10   find use in a clinical setting. Estrogen receptor is a
DNA-binding protein, the dysfunction of which has been
implicated in the etiology of breast cancer. Described
herein is a method which could be employed to assay for
functional estrogen receptor in biopsies from breast
15   tissue.

A cell extract will be prepared from the biopsied
tissue by gentle homogenization and centrifugation as
described by Flanagan et al. (19). A double-stranded
oligodeoxyribonucleotide which forms a consensus
20   estrogen response element (ERE) can be prepared by
automated synthesis of a pair of oligonucleotides. A
preferred sequence for the sense strand is

      5'-F-GGTCACTGTGACC-3'

wherein the fluorophore (F) is incorporated by use of
25   fluorescently-labelled phosphoramidite linker in the
final nucleotide addition step of the synthesis. This
oligonucleotide is self-complementary. Annealing of the
oligonucleotide in solution provides a double-stranded
oligonucleotide labelled with a fluorphore at each 5'
30   end (*ERE).

The buffer for conducting the fluorescence
polarization measurements is 10% glycerol, 80 mM KCl, pH
7.9 containing 0.2 mM EDTA. Measurements are made at
room temperature.

35   Preliminary measurements are made of the
fluorescence intensity of the cell extract containing no

21

ERE oligonucleotide (background).   These values are
subtracted from the measurements used in determining the
emission polarization of those samples which contain
cell extract.   Preliminary measurements are also made of
5   the   fluorescence   polarization   of   the   *ERE
oligodeoxyribonucleotide in a solution free of the cell
extract.   Then aliquots of the cell extract are mixed
with 1 pM of the *ERE oligodeoxyribonucleotide and the
fluorescence polarization is again measured. Comparison
10   of the polarization values obtained in the presence of
cell extract with the value of the free *ERE provides a
measure of the binding function of estrogen receptor
present in the sample.

As a control experiment, results using a sample
15   cell extract prepared from cells known to contain
functional estrogen receptor should be obtained and
compared with the results from the extract prepared from
the clinical sample.

To most clearly describe the experiment, an example
20   of sample formulations is provided in Table II.

Table II

SAMPLE FORMULATIONS FOR DETERMINING BINDING
OF ERE TO ESTROGEN RECEPTOR
IN A CLINICAL SAMPLE

	Biopsy			Background		
[ERE]	*ERE	Extract	Buffer	*ERE	Extract	Buffer
1pM	5[a]	0	995	0	0	1000
1pM	5	100	895	0	100	900
1pM	5	250	745	0	250	750
1pM	5	500	455	0	500	500
1pM	5	950	45	0	950	50

	Control			Background		
[ERE]	*ERE	Extract	Buffer	*ERE	Extract	Buffer
1pM	5	0	995	0	0	1000
1pM	5	100	895	0	100	900
1pM	5	250	745	0	250	750
1pM	5	500	455	0	500	500
1pM	5	950	45	0	950	50

[a] All amounts are in μl.

To quantitate the amount of estrogen receptor in the sample, the cell extract can be titrated with various amounts of labelled *ERE and the result compared with a standard curves obtained using known amounts of purified estrogen receptor and *ERE.

Example 7: Clinical assay for a specific point mutation in a gene by oligonucl otide hybridization to mRNA from a tissue biopsy

Specific mutations in the cholesterol receptor gene

23

have been found to lead to hypercholesterolemia, a
condition which leads to early death of the patient due
to arterial disease if measures are not taken to control
cholesterol levels in the circulation. It is expected
5   that early detection of the condition by neonatal or
prenatal genetic testing, and thus early intervention
would provide for a better prognosis for afflicted
individuals. Accordingly, in this example, a method for
detecting mutations in the cholesterol receptor gene by
10   assay of mRNA for binding to oligonucleotide probes is
described. This method is of course generalizable to
the detection of other mutations.

      8-10 base oligonucleotides, labelled on the 5' end
by methods known in the art, are designed to be
15   complementary to those portions of the mRNA transcript
of the wild type cholesterol receptor gene which have
been previously shown to be mutated in patients having
familial hypercholesterolemia. Each oligonucleotide is
designed to provide a similar decrease in T_m (the melting
20   temperature of a duplex between the oligo and a
hybridized complementary strand) upon binding to a
sequence that is entirely complementary, with the
exception of a single mismatched base pair, by varying
its length and GC-content. An experiment is conducted
25   to determine the temperature at which the wild type mRNA
remains mostly hybridized to each oligonucleotide, but
mRNA obtained from a liver biopsy of a panel of patients
which present the mutations observed to cause
hypercholesterolemia is not hybridized by the
30   oligonucleotide complementary to the wild type sequence
in the mutated region.

      100 mg of tissue generally yields approximately 1
pmole of a specific 1 kilobase mRNA. In a volume of 1
ml, this would correspond to 1 nM of specific mRNA. For
35   a 1:1 hybridization, 1 nM fluorescently labelled
oligonucleotide would be used. This sample size
provides a readily detected signal; thus smaller samples

24

could be used. The fluorescence polarization of the oligonucleotide alone, mixed with wild-type mRNA at 1 nM and mixed with mRNA from the clinical sample at approximately 1 nM are each determined at the

5   temperature found in the initial experiment to provide the maximal difference in binding of the oligonucleotide to the wild-type and the mutant mRNA sequence. It would be observed that tissue containing the wild-type mRNA would show a polarization greater than that observed for

10   the oligonucleotide alone, while tissue containing mutant mRNA would show a polarization equal to that of the oligonucleotide alone.

As a control experiment, the fluorescence emission of the mRNA preparation in the absence of labelled

15   oligonucleotide is determined in each plane of emission polarization. These background values are subtracted from each measurement made in the hybridization experiments.

Example 8:   Apparatus for conducting fluorescence

20   **polarization measurements with high sensitivity**

The diagram shown in Figure 5 shows modifications made to an ISS Koala™ fluorometer to produce an instrument having the sensitivity required to implement the present invention. The modifications include (1)

25   reducing the light path from 30 cm to 20 cm; (2) positioning the emission lens to within 1 cm of the sample holder; and (3) replacing the Glan Thompson polarizers with UV grade film polarizers mounted directly on the sample holder. These improvements

30   increase the instrument sensitivity dramatically. In the tests done to demonstrate the increase in sensitivity achieved by these modifications, a fluorescently labeled 25 mer oligonucleotide with the following sequence was used.

35   5'-F-ATCGAACTAGTTAACTAGTACGCAA-3'

25

As shown in Table III, we were able to detect oligonucleotide at a concentration of 9 pM in 500 μl using a Xenon arc lamp excitation source rather than the argon ion source indicated in the drawing. This
5   represents an increase in sensitivity of 10-fold as compared to the unmodified instrument. Given that we can detect a 10% change in the polarization, we can therefore detect 0.9 pM of protein or nucleic acid or synthetic polymer in solution. This level of
10 sensitivity corresponds to 450 attomoles of macromolecule.

Table III
SENSITIVITY LIMIT ASSAY
USING MODIFIED FLUOROMETER AND SAMPLE CELL

Solution	Intensity (Counts)
9 pM F-25 mer	1967.8 +/- 1.2
Buffer background	243.9 +/- 0.4

Sensitivity of the modified instrument could be improved by two further modifications. (1) Including a
20 sharp bandpass excitation filter would increase sensitivity by a factor of 10-fold. (2) Substitution of an argon ion laser light source for the Xenon arc lamp used in the test reported herein would increase sensitivity by an additional 100-fold due to the
25 increased intensity of the excitation beam and its monochromatic and polarized nature. Since we are background limited and the monochromators in the basic ISS instrument are not good at stray light rejection, most of the background intensity results from scattered
30 exciting light. With a laser, there is only 488 nm light, and thus no stray light of other wavelengths will enter the sample. Secondly, laser light is plane polarized, eliminating the need for an excitation polarizer. Finally, fluorescence intensity is directly
35 proportional to the excitation source intensity, and a

26

laser, even a small one, would provide more power than
the Xenon arc lamp. In the modified design, dilution
with concomitant increase in gain did not increase
sensitivity, since use of a lamp excitation source
5   caused the detection level to be background-limited
rather than noise-limited. Thus, another factor of 10
increase in sensitivity is available if the stray
exciting light can be eliminated.

The invention being thus described, various
10 modifications of the materials and methods set forth in
the description will be obvious to a practitioner of the
invention who is skilled in the art. Such variations
upon the invention described are to be considered within
the scope of the invention as claimed below.

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disclosure. Each of the references below is hereby
incorporated in its entirety by such citation.
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28

CLAIMS

What is claimed is:

1.   1.   A method for measuring the binding of a
2   polynucleotide to a macromolecule to form a non-covalent
3   complex which comprises:
4   i) labelling said polynucleotide with a fluorescent
5   label;
6   ii) obtaining a first measurement of the
7   polari.ation of the fluorescent emission from said
8   label;
9   ii.) contacting said labelled polynucleotide with
10   said macromolecule, under conditions favorable to
11   binding of said labelled polynucleotide to said
12   macromolecule;
13   iv) obtaining a second measurement of the
14   polarization of the fluorescent emission from said label
15   under said conditions favorable to binding; and
16   v) comparing said first measurement with said
17   second measurement.

1   2.   A method as recited in claim 1, wherein said
2   label is fluorescein.

1   3.   A method as recited in claim 1, wherein said
2   polynucleotide   is   a   single-stranded
3   oligodeoxyribonucleotide of length less than or equal to
4   40 bases.

29

1   4.   A method as recited in claim 1, wherein said
2   polynucleotide   is   a   double-stranded
3   oligodeoxyribonucleotide of length less than or equal to
4   40 basepairs.

1   5.   A method as recited in claim 1, wherein said
2   macromolecule is a protein.

1   6.   A method as recited in claim 3, wherein said
2   macromolecule is a second polynucleotide.

1   7.   A method as recited in claim 1, wherein said
2   polynucleotide is present at a concentration of less
3   than or equal to 1 nanomolar.

1   8.   A method as recited in claim 7, wherein said
2   macromolecule is present at a concentration of less than
3   or equal to 1 nanomolar.

1   9.   A method for diagnosing a genetic mutation
2   which comprises:
3   i) obtaining a sample of DNA from a patient;
4   ii)   obtaining   a   first   measurement   of   the
5   polarization   of   the   fluorescent   emission   from   a
6   fluorescently-labelled oligonucleotide;
7   iii)   contacting   said   fluorescently-labelled
8   oligonucleotide with said DNA under conditions favorable
9   to   binding   of   said   fluorescently-labelled
10   oligonucleotide to said DNA;
11   iv)   obtaining   a   second   measurement   of   the
12   polarization   of   the   fluorescent   emission   from   said
13   fluorescently-labelled   oligonucleotide   under   said
14   conditions favorable to binding; and
15   v) comparing said second measurement to said first
16   measurement.

30

1   10.   A method for diagnosing variation in the
2   amount or nature of a DNA binding protein which
3   comprises:
4   i) fluorescently labelling an oligonucleotide
5   having the nucleotide sequence recognized by a DNA-
6   binding protein, wherein said oligonucleotide may be
7   either double-stranded or single stranded.
8   ii) obtaining a sample containing the DNA-binding
9   protein to be tested;
10   iii) obtaining a first measurement of the
11   polarization of the fluorescent emission from said
12   fluorescently-labelled oligonucleotide;
13   iv) contacting said fluorescently-labelled
14   oligonucleotide with said sample under conditions
15   favorable to binding of said fluorescently-labelled
16   polynucleotide to said DNA-binding protein;
17   v) obtaining a second measurement of the
18   polarization of the fluorescent emission from said
19   fluorescently-labelled oligonucleotide under said
20   conditions favorable to binding; and
21   vi) comparing said second measurement to said first
22   measurement.

1   11.   The method of claim 8, which further
2   comprises:
3   vii) adding a second aliquot of unlabelled
4   polynucleotide having the same nucleotide sequence as
5   said labelled polynucleotide;
6   viii) obtaining a third measurement of the
7   polarization of the fluorescent emission from said
8   fluorescently-labelled oligonucleotide under said
9   conditions favorable to binding; and
10   vi) comparing said measurement to said first and
11   second measurements.

1   12. An apparatus for measuring the polarization of
2 fluorescent emission which comprises:
3   a box for the exclusion of light, in which is
4 placed;
5   an excitation means for producing polarized light
6 having a narrow range of wavelengths;
7   a sample chamber illuminated by said polarized
8 light;
9   emission polarizers for imposing polarization upon
10 fluorescence emission from said sample chamber, wherein
11 said emission polarizers are of a film type;
12   detection means for detecting said fluorescence
13 emission; and
14   emission lenses for focussing said fluorescent
15 emission upon said detection means.

1   13. An apparatus for measuring the polarization of
2 fluorescent emission which comprises:
3   a box for the exclusion of light in which is placed
4 an illumination means so as to illuminate a sample
5 chamber, through a collimating lens and a focusing lens,
6 and further through a first polarizing means for plane
7 polarizing the light from said illumination means, said
8 lenses and first polarizing means, being mounted in a
9 line connecting said illumination means and said sample
10 chamber, and further said sample chamber being mounted
11 between two additional polarizing means, said polarizing
12 means being placed so that the line between them and
13 including said sample chamber is perpendicular to the
14 light path from said illumination means to said sample
15 chamber, said polarizing means being mounted so that the
16 plane of polarization of the second of said polarizing
17 means is parallel to the plane of polarization of said
18 first polarizing means and also so that the plane of
19 polarization of said second polarizing means is
20 perpendicular to the plane of polarization of the third
21 of said polarizing means and in which box is also placed

32

22   detection means for the collection and quantitation of
23   the amount of light which passes through said polarizing
24   means from said sample chamber to said detecting means.

1   14.   The apparatus of claim 12, wherein said
2   illumination means is a xenon arc lamp.

1   15.   The apparatus of claim 13, wherein said
2   collimating lens and said focusing lens are substituted
3   by a fiber optic cable.

1   16.   The apparatus of claim 13, wherein said
2   illumination means is a laser and each of said
3   collimating lens, said focusing lens and said first
4   polarizing means are not present.

1   17. The apparatus of claim 16, wherein said laser
2   is a tunable dye laser or a solid state laser.

1   18.   The apparatus of claim 13, wherein each of
2   said polarizing means are ultraviolet grade film
3   polarizers.

1   19.   The apparatus of claim 16, wherein each of
2   said polarizing means are ultraviolet grade film
3   polarizers.

1   20.   The apparatus of claim 13, wherein the length
2   of the light path from said illumination means to said
3   detection means is 20 centimeters or less.

1   21.   The apparatus of claim 16, wherein the light
2   from said illumination means is conducted to said sample
3   chamber by a glass fiber.

1   22. An apparatus for measuring the polarization of
2 fluorescent emission which comprises:
3   A box for the exclusion of light in which is placed
4 an illumination means so as to illuminate a sample
5 chamber through an excitation lens and further through
6 an excitation filter, wherein said excitation filter
7 passes only a narrow range of wavelengths of light, and
8 further through a fixed means of plane polarizing light
9 which shines on said sample chamber, such that each of
10 these elements are mounted in the light path from said
11 illuminating means to said sample chamber, said sample
12 chamber being mounted relative to a rotatable polarizing
13 means, which plane polarizes light which passes through
14 said rotatable plane polarizing means, so that a path
15 from said illumination means through said sample chamber
16 to said rotatable polarizing means forms a right angle
17 with the sample chamber at the vertex and in which box
18 is also placed an emission filter interposed between the
19 sample chamber and said rotatable plane polarizing
20 means, wherein said emission filter will allow only a
21 narrow range of wavelengths of light to pass and in
22 which box is further mounted detection means for the
23 collection and quantitation of the amount of light which
24 passes through said rotatable polarizing means from said
25 sample chamber to said detecting means.

1   23.   The apparatus of claim 22, wherein said
2 illumination means is a laser and each of said
3 excitation lens, excitation filter and said fixed
4 polarization means are not present.

1   24. A kit for the clinical diagnosis of the amount
2 and function of a specific DNA-binding protein, which
3 comprises a fluorescently labelled oligonucleotide and
4 a container.

34

1   25.   The   kit   of   claim   24,   wherein   said

2   oligonucleotide has the sequence

3   5'-GGTCACTGTGACC-3'

35

AMENDED CLAIMS
[received by the International Bureau on 19 April 1994 (19.04.94);
 original claims 1-25 replaced by amended claims 1-30 (11 pages)]

What is claimed is:

1   1. A method for measuring the binding of a
2   polynucleotide to a macromolecule to form a complex
3   which comprises:

4   i) labelling said polynucleotide with a fluorescent
5   label;

6   ii)   obtaining   a   first   measurement   of   the
7   polarization of the fluorescent emission from said
8   label;

9   iii) contacting said labelled polynucleotide with
10   said   macromolecule,   under   conditions   favorable   to
11   binding of said polynucleotide to said macromolecule and
12   such   that   the   concentration   of   said   labelled
13   polynucleotide is less than or equal to 5 nanomolar;

14   iv)   obtaining   a   second   measurement   of   the
15   polarization of the fluorescent emission from said
16   · label; and

17   v) comparing said first measurement with said
18   second measurement.

1   2.   A method as recited in claim 1, wherein said
2   label is fluorescein.

1   3.   A method as recited in claim 1, wherein said
2   polynucleotide   is   a   single-stranded
3   oligodeoxyribonucleotide of length less than or equal to
4   40 bases.

1   4.   A method as recited in claim 1, wherein said
2 polynucleotide   is   a   double-stranded
3 oligodeoxyribonucleotide of length less than or equal to
4 40 basepairs.

1   5.   A method as recited in claim 1, wherein said
2 macromolecule is a protein.

1   6.   A method as recited in claim 3, wherein said
2 macromolecule is a second polynucleotide.

1   7.   A method as recited in claim 1, wherein said
2 macromolecule is present at a concentration of less than
3 or equal to 1 nanomolar.

1   8.   A method for diagnosing a genetic mutation
2 which comprises:
3   i) obtaining a sample of DNA from a patient;
4   ii)   obtaining   a   first   measurement   of   the
5 polarization   of   the   fluorescent   emission   from   a
6 fluorescently-labelled oligonucleotide;
7   iii)   contacting   said   fluorescently-labelled
8 oligonucleotide with said DNA under conditions such that
9 the   concentration   of   said   fluorescently-labelled
10 oligonucleotide is less than 5 nanomolar;
11   iv)   obtaining   a   second   measurement   of   the
12 polarization   of   the   fluorescent   emission   from   said
13 fluorescently-labelled oligonucleotide; and
14   v) comparing said second measurement to said first
15 measurement.

AMENDED SHEET (ARTICLE 19)

37

1   9. A method for diagnosing variation in the amount
2   or nature of a DNA binding protein which comprises:
3   i) fluorescently labelling an oligonucleotide
4   having the nucleotide sequence recognized by a DNA-
5   binding protein, wherein said oligonucleotide may be
6   either double-stranded or single stranded.
7   ii) obtaining a sample containing the DNA-binding
8   protein to be tested;
9   iii) obtaining a first measurement of the
10 polarization of the fluorescent emission from said
11 fluorescently-labelled oligonucleotide;
12   iv) contacting said fluorescently-labelled
13 oligonucleotide with said sample under conditions such
14 that the concentration of said fluorescently-labelled
15 polynucleotide is less than 5 nanomolar;
16   v) obtaining a second measurement of the
17 polarization of the fluorescent emission from said
18 fluorescently-labelled oligonucleotide;
19   vi) comparing said second measurement to said first
20 measurement; and
21   vii) diagnosing a variation in the amount or nature
22 of said DNA binding protein by observing a change in the
23 polarization measured in step (iii) compared to the
24 polarization measured in step (vi).

1   10.   The method of claim 7, which further
2   comprises:
3   vii) adding a second aliquot of unlabelled
4   polynucleotide having the same nucleotide sequence as
5   said labelled polynucleotide;
6   viii) obtaining a third measurement of the
7   polarization of the fluorescent emission from said
8   fluorescently-labelled oligonucleotide under said
9   conditions favorable to binding; and
10   vi) comparing said measurement to said first and
11 second measurements.

AMENDED SHEET (ARTICLE 19)

38

1   11. An apparatus for measuring the polarization of
2 fluorescent emission which comprises:
3   a box for the exclusion of light, in which is
4 placed;
5   an excitation means for producing polarized light
6 having a narrow range of wavelengths;
7   a sample chamber illuminated by said polarized
8 light;
9   emission polarizers for imposing polarization upon
10 fluorescence emission from said sample chamber, wherein
11 said emission polarizers are of a film type;
12   detection means for detecting said fluorescence
13 emission; and
14   emission lenses for focussing said fluorescent
15 emission upon said detection means.

1   12. An apparatus for measuring the polarization of
2 fluorescent emission which comprises:
3   a box for the exclusion of light in which is placed
4 an illumination means so as to illuminate a sample
5 chamber, through a collimating lens and a focusing lens,
6 and further through a first polarizing means for plane
7 polarizing the light from said illumination means, said
8 lenses and first polarizing means, being mounted in a
9 line connecting said illumination means and said sample
10 chamber, and further said sample chamber being mounted
11 between two additional polarizing means, said polarizing
12 means being placed so that the line between them and
13 including said sample chamber is perpendicular to the
14 light path from said illumination means to said sample
15 chamber, said polarizing means being mounted so that the
16 plane of polarization of the second of said polarizing
17 means is parallel to the plane of polarization of said
18 first polarizing means and also so that the plane of
19 polarization of said second polarizing means is
20 perpendicular to the plane of polarization of the third
21 of said polarizing means and in which box is also placed

AMENDED SHEET (ARTICLE 19)

22   detection means for the collection and quantitation of
23   the amount of light which passes through said polarizing
24   means from said sample chamber to said detecting means.

1   13.   The apparatus of claim 11, wherein said
2   illumination means is a xenon arc lamp.

1   14.   The apparatus of claim 11, wherein said
2   collimating lens and said focusing lens are substituted
3   by a fiber optic cable.

1   15.   The apparatus of claim 12, wherein said
2   illumination means is a laser and each of said
3   collimating lens, said focusing lens and said first
4   polarizing means are not present.

1   16.   The apparatus of claim 15, wherein said laser
2   is a tunable dye laser or a solid state laser.

1   17.   The apparatus of claim 12, wherein each of
2   said polarizing means are ultraviolet grade film
3   polarizers.

1   18.   The apparatus of claim 15, wherein each of
2   said polarizing means are ultraviolet grade film
3   polarizers.

1   19.   The apparatus of claim 12, wherein the length
2   of the light path from said illumination means to said
3   detection means is 20 centimeters or less.

1   20.   The apparatus of claim 15, wherein the light
2   from said illumination means is conducted to said sample
3   chamber by a glass fiber.

40

1   21. An apparatus for measuring the polarization of
2   fluorescent emission which comprises:
3   A box for the exclusion of light in which is placed
4   an illumination means so as to illuminate a sample
5   chamber through an excitation lens and further through
6   an excitation filter, wherein said excitation filter
7   passes only a narrow range of wavelengths of light, and
8   further through a fixed means of plane polarizing light
9   which shines on said sample chamber, such that each of
10   these elements are mounted in the light path from said
11   illuminating means to said sample chamber, said sample
12   chamber being mounted relative to a rotatable polarizing
13   means, which plane polarizes light which passes through
14   said rotatable plane polarizing means, so that a path
15   from said illumination means through said sample chamber
16   to said rotatable polarizing means forms a right angle
17   with the sample chamber at the vertex and in which box
18   is also placed an emission filter interposed between the
19   sample chamber and said rotatable plane polarizing
20   means, wherein said emission filter will allow only a
21   narrow range of wavelengths of light to pass and in
22   which box is further mounted detection means for the
23   collection and quantitation of the amount of light which
24   passes through said rotatable polarizing means from said
25   sample chamber to said detecting means.

1   22.   The apparatus of claim 21, wherein said
2   illumination means is a laser and each of said
3   excitation lens, excitation filter and said fixed
4   polarization means are not present.

1   23. A kit for the clinical diagnosis of the amount
2   and function of a specific DNA-binding protein, which
3   comprises a fluorescently labelled oligonucleotide and
4   a container.

41

1   24.   The kit of claim 23, wherein said
2   oligonucleotide has the sequence
3   5'-GGTCACTGTGACC-3'

1   25. A method for measuring the binding of a
2   polynucleotide to a macromolecule to form a complex
3   which comprises:
4   i) labelling said polynucleotide with a fluorescent
5   label;
6   ii) obtaining a first measurement of the
7   polarization of the fluorescent emission from said
8   label;
9   iii) contacting said labelled polynucleotide with
10   said macromolecule, under conditions favorable to
11   binding of said polynucleotide to said macromolecule and
12   such that the concentration of said labelled
13   polynucleotide ranges from 1 nanomolar to 10 picomolar;
14   iv) obtaining a second measurement of the
15   polarization of the fluorescent emission from said
16   label; and
17   v) comparing said first measurement with said
18   second measurement.

1   26.   A method for diagnosing a genetic mutation
2   which comprises:
3   i) obtaining a sample of DNA from a patient;
4   ii) obtaining a first measurement of the
5   polarization of the fluorescent emission from a
6   fluorescently-labelled oligonucleotide;
7   iii) contacting said fluorescently-labelled
8   oligonucleotide with said DNA under conditions such that
9   the concentration of said fluorescently-labelled
10   oligonucleotide ranges from 1 nanomolar to 10 picomolar;
11   iv) obtaining a second measurement of the
12   polarization of the fluorescent emission from said
13   fluorescently-labelled oligonucleotide;
14   v). comparing said second measurement to said first

42

15   measurement; and

16   vi) diagnosing a genetic mutation by observing a

17   change in polarization measured in step (ii) and step

18   (iv).

1   27.   A method for diagnosing variation in the

2   amount or nature of a DNA binding protein which

3   comprises:

4   i) fluorescently labelling an oligonucleotide

5   having the nucleotide sequence recognized by a DNA-

6   binding protein, wherein said oligonucleotide may be

7   either double-stranded or single stranded.

8   ii) obtaining a sample containing the DNA-binding

9   protein to be tested;

10   iii) obtaining a first measurement of the

11   polarization of the fluorescent emission from said

12   fluorescently-labelled oligonucleotide;

13   iv)   contacting said fluorescently-labelled

14   oligonucleotide with said sample under conditions such

15   that the concentration of said fluorescently-labelled

16   polynucleotide ranges from 1 nanomolar to 10 picomolar;

17   v)   obtaining a second measurement of the

18   polarization of the fluorescent emission from said

19   fluorescently-labelled oligonucleotide;

20   vi) comparing said second measurement to said first

21   measurement; and

22   vii) diagnosing a variation in the amount or nature

23   of said DNA binding protein by observing a change in the

24   polarization measured in step (iii) compared to the

25   polarization measured in step (vi).

43

1   28. A method for measuring the binding of a
2   polynucleotide to a macromolecule to form a complex
3   which comprises:
4   i) labelling said polynucleotide with a fluorescent
5   label;
6   ii) obtaining a first measurement of the
7   polarization of the fluorescent emission from said
8   label;
9   iii) contacting said labelled polynucleotide with
10   said macromolecule, under conditions favorable to
11   binding of said polynucleotide to said macromolecule and
12   such that the concentration of said labelled
13   polynucleotide ranges from 1 nanomolar to 10 picomolar;
14   iv) obtaining a second measurement of the
15   polarization of the fluorescent emission from said
16   label;
17   v) comparing said first measurement with said
18   second measurement; and
19   vi) detecting formation of said complex by
20   observing an increase in the polarization measured in
21   step (iv) compared to the polarization measured in step
22   (ii);
23   wherein said measurements of fluorescence polarization
24   are made using an apparatus having a component selected
25   from the group consisting of a UV grade film polarizer
26   mounted directly on the sample compartment, a Xenon arc
27   lamp excitation source, a Xenon arc lamp excitation
28   source coupled to the sample compartment by a fiber
29   optic cable, and a laser light source.

1　　29.　A method for diagnosing a genetic mutation
2　which comprises:
3　　　　i) obtaining a sample of DNA from a patient;
4　　　　ii) obtaining a first measurement of the
5　polarization of the fluorescent emission from a
6　fluorescently-labelled oligonucleotide;
7　　　　iii) contacting said fluorescently-labelled
8　oligonucleotide with said DNA under conditions such that
9　the concentration of said fluorescently-labelled
10　oligonucleotide ranges from 1 nanomolar to 10 picomolar;
11　　　　iv) obtaining a second measurement of the
12　polarization of the fluorescent emission from said
13　fluorescently-labelled oligonucleotide;
14　　　　v) comparing said second measurement to said first
15　measurement; and
16　　　　vi) diagnosing a genetic mutation by observing a
17　change in polarization measured in step (ii) and step
18　(iv);
19　wherein said measurements of fluorescence polarization
20　are made using an apparatus having a component selected
21　from the group consisting of a UV grade film polarizer
22　mounted directly on the sample compartment, a Xenon arc
23　lamp excitation source, a Xenon arc lamp excitation
24　source coupled to the sample compartment by a fiber
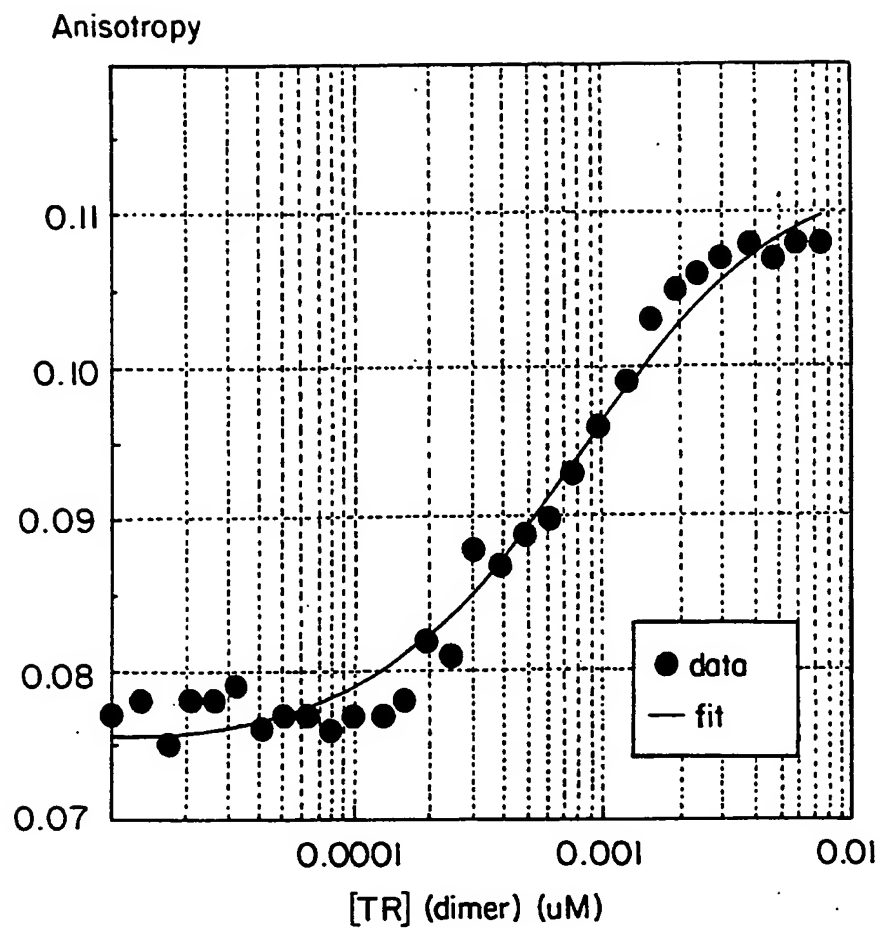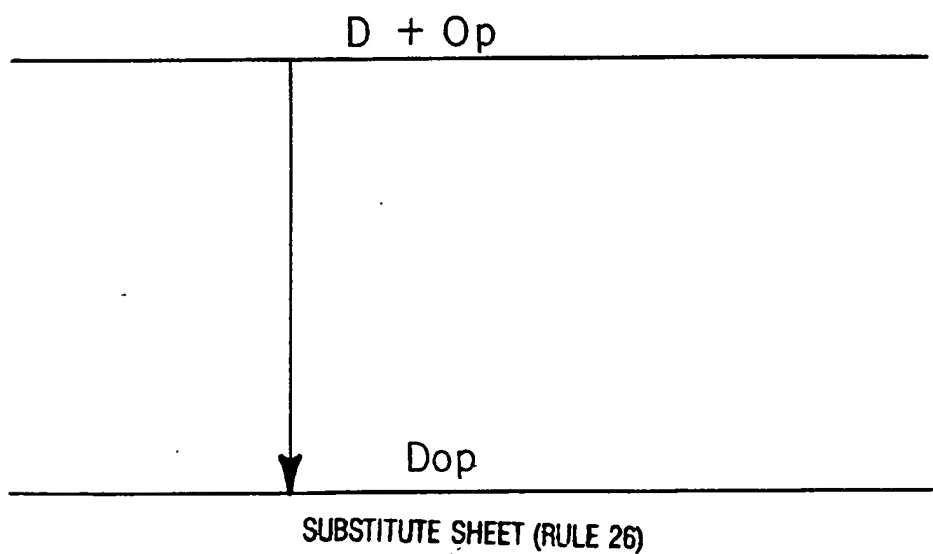25　optic cable, and a laser light source.

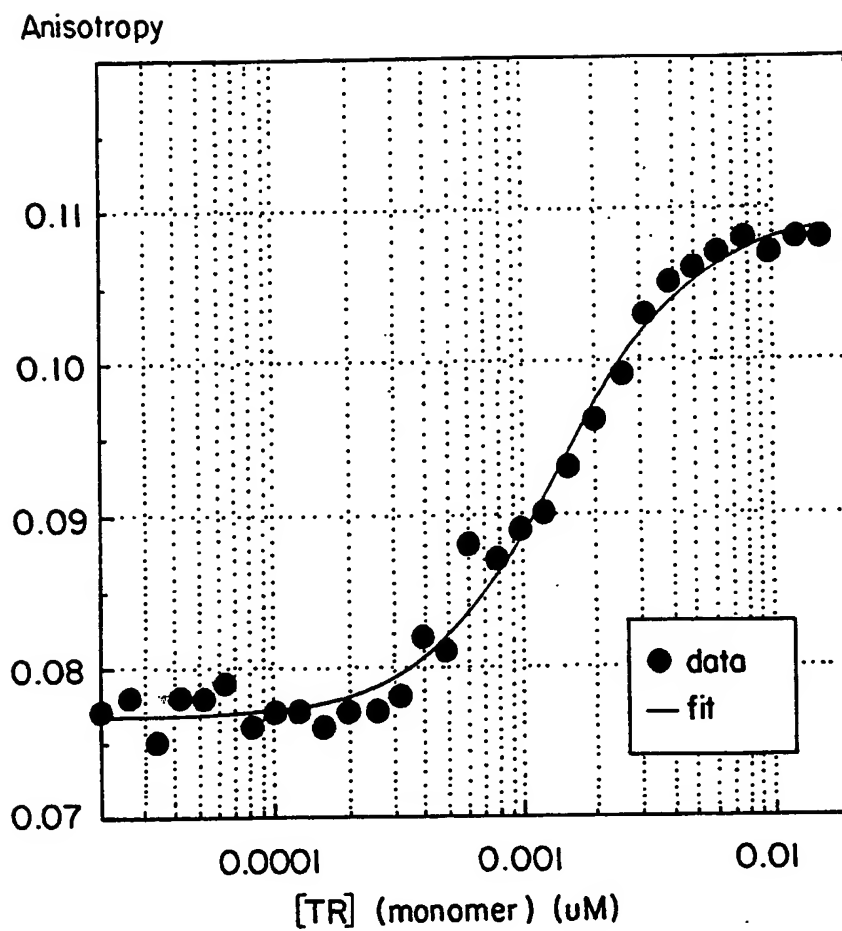1   30.   A method for diagnosing variation in the
2   amount or nature of a DNA binding protein which
3   comprises:
4   i) fluorescently labelling an oligonucleotide
5   having the nucleotide sequence recognized by a DNA-
6   binding protein, wherein said oligonucleotide may be
7   either double-stranded or single stranded.
8   ii) obtaining a sample containing the DNA-binding
9   protein to be tested;
10   iii) obtaining a first measurement of the
11 polarization of the fluorescent emission from said
12 fluorescently-labelled oligonucleotide;
13   iv) contacting said fluorescently-labelled
14 oligonucleotide with said sample under conditions such
15 that the concentration of said fluorescently-labelled
16 polynucleotide ranges from 1 nanomolar to 10 picomolar;
17   v) obtaining a second measurement of the
18 polarization of the fluorescent emission from said
19 fluorescently-labelled oligonucleotide;
20   vi) comparing said second measurement to said first
21 measurement; and
22   vii) diagnosing a variation in the amount or nature
23 of said DNA binding protein by observing a change in the
24 polarization measured in step (iii) compared to the
25 polarization measured in step (vi).;
26 wherein said measurements of fluorescence polarization
27 are made using an apparatus having a component selected
28 from the group consisting of a UV grade film polarizer
29 mounted directly on the sample compartment, a Xenon arc
30 lamp excitation source, a Xenon arc lamp excitation
31 source coupled to the sample compartment by a fiber
32 optic cable, and a laser light source.

FIG. I A

Anisotropy

[TR] (dimer) (uM)

FIG. IC

D + Op

Dop

FIG. IB

Anisotropy

[TR] (monomer) (uM)

FIG. ID

$2M + Op$

MOp

M2

M2Op

FIG. 2

FIG. 3

ANISOTROPY

[TR]   (dimer nM)

FIG. 4

ANISOTROPY

[TR] (dimer nM)
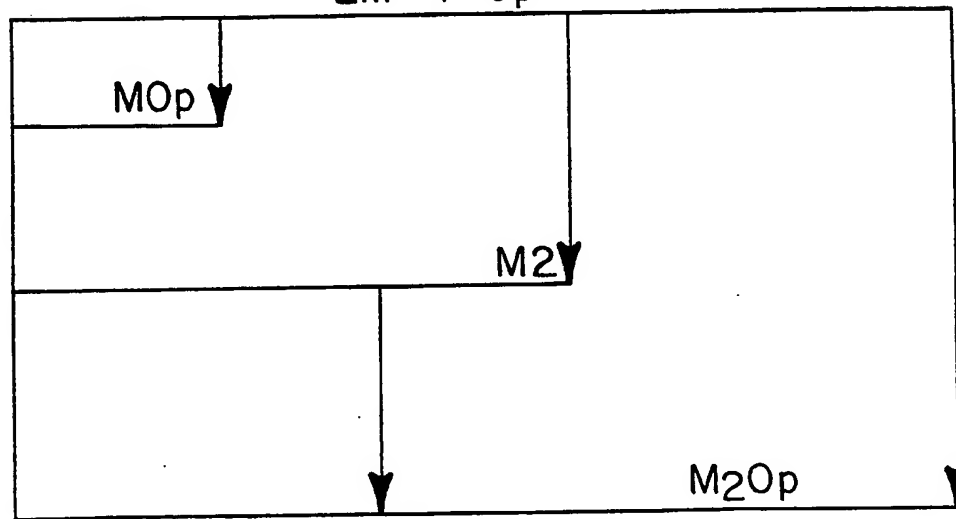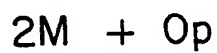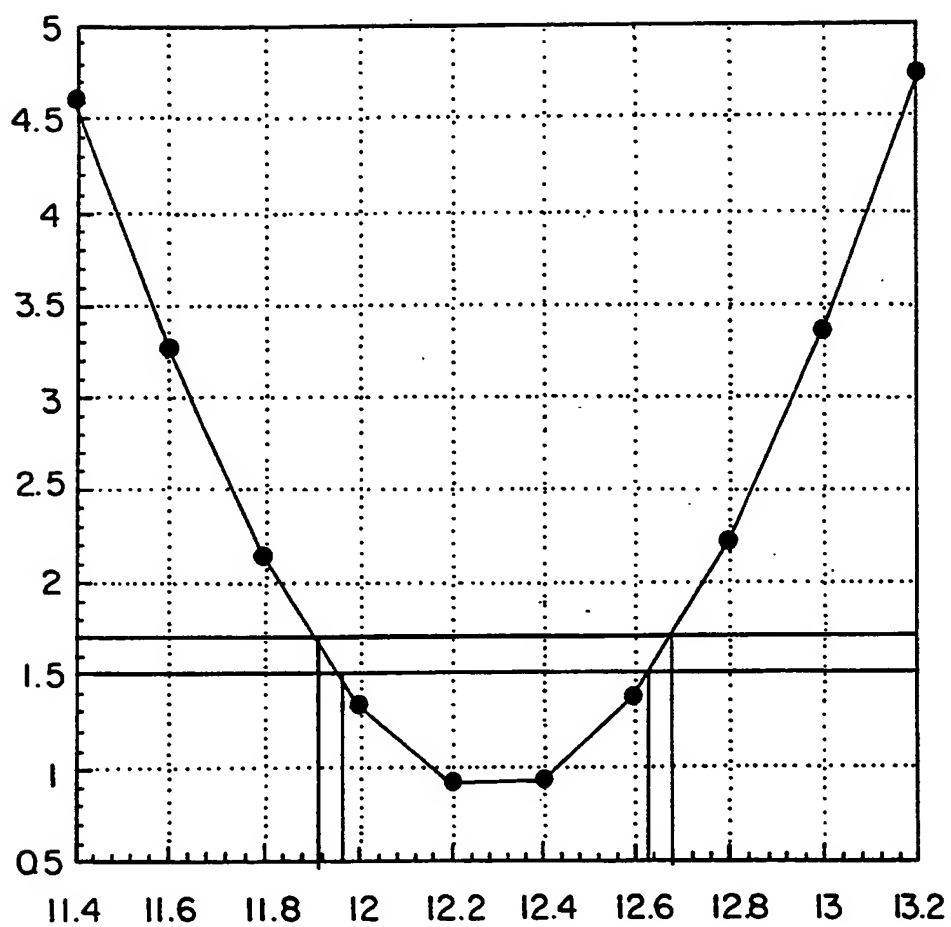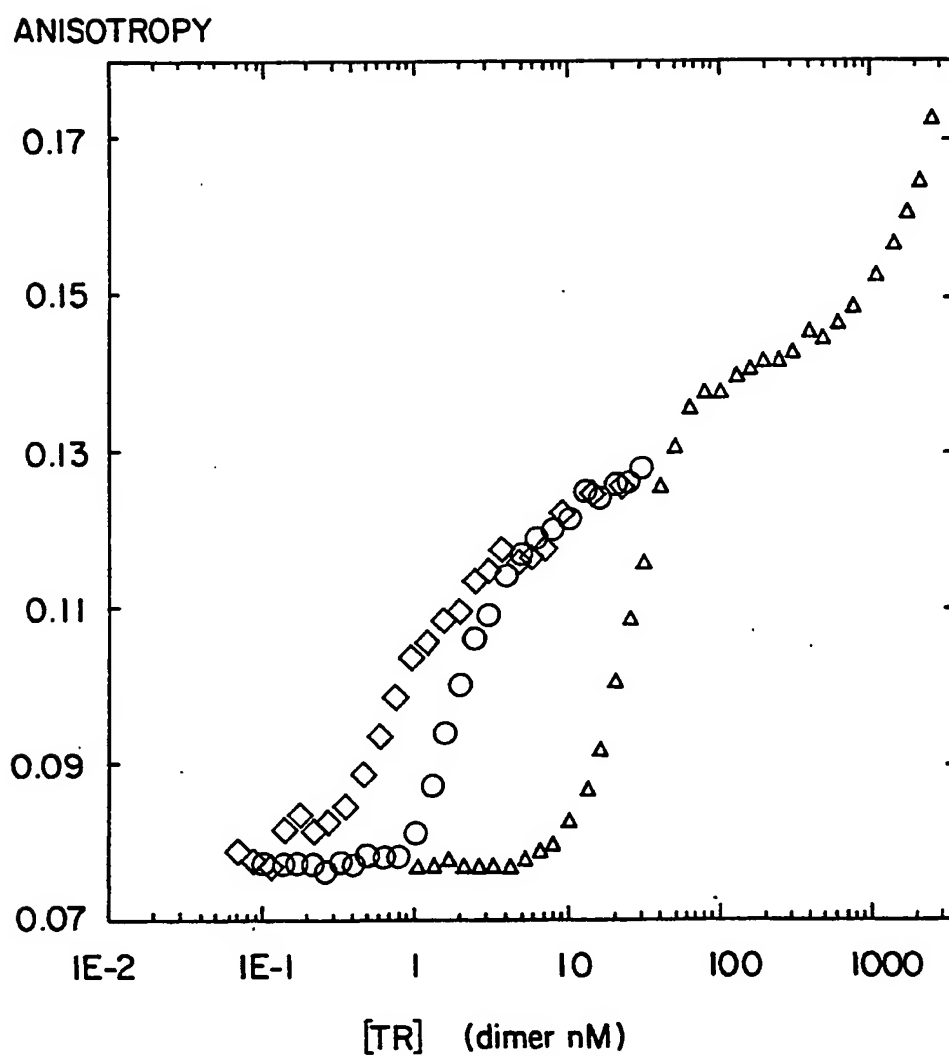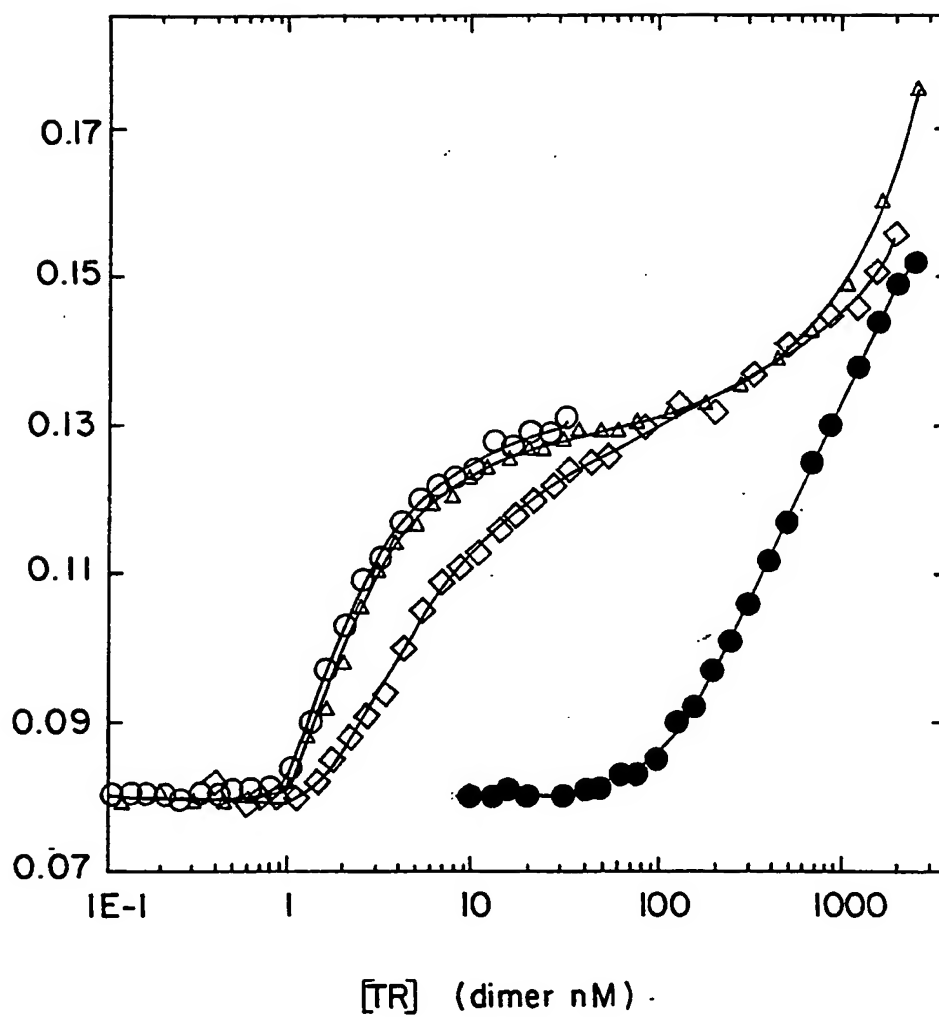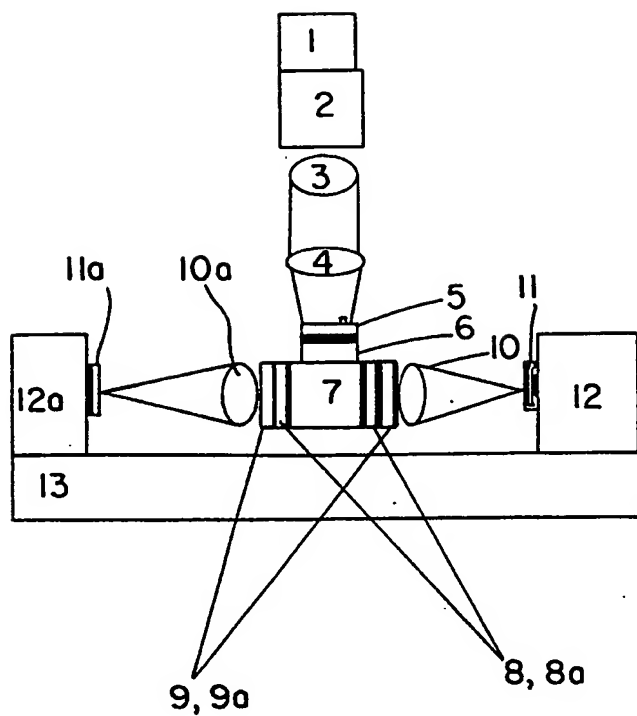
FIG. 5
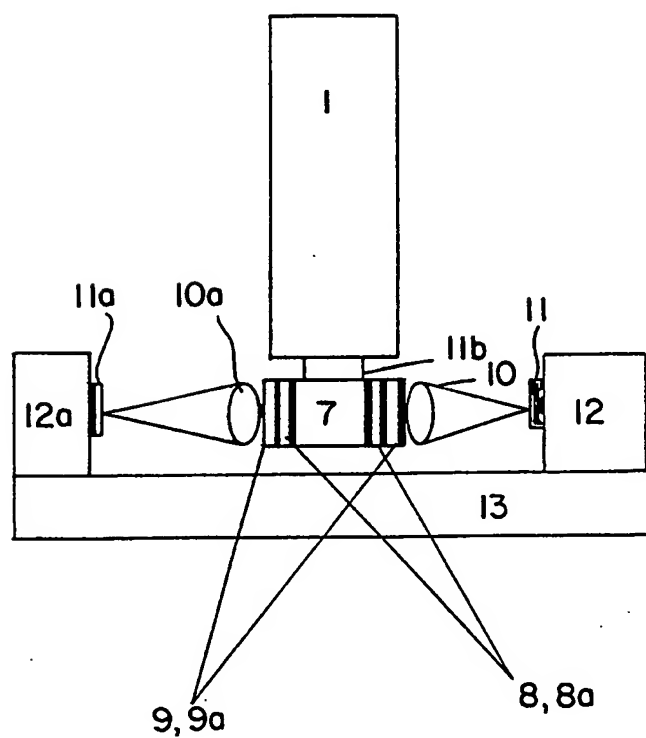
FIG. 6

FIG.7
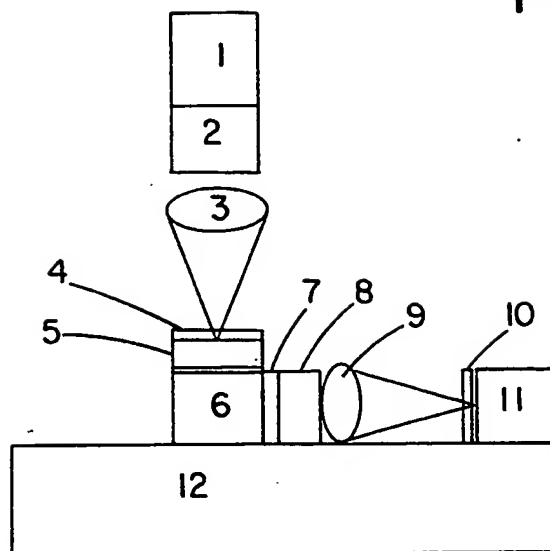
FIG.8

FIG.9

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5)   :C12Q 1/68; C12M 1/34; G01J 1/42
US CL   :436/6, 291; 250/372
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   436/6, 291; 250/372

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, APS

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Vol. 19, No. 15, issued 1991, Murakami et al., "Fluorescent-labeled oligonucleotide probes: detection of hybrid formation in solution by fluorescence polarization spectroscopy," pages 4097-4102, see entire document.	1-25
Y	Proceedings of the National Academy of Sciences, Vol. 87, issued March 1990, Heyduk et al., "Application of fluorescence energy transfer and polarization to monitor *Escherichia coli* cAMP receptor protein and *lac* promoter interaction," pages 1744-1748, see entire document.	1-25

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 February 1994	**16 FEB 1994**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SCOTT HOUTTEMAN
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Analytical Biochemistry, Vol. 169, issued 1988, Matthews et al., "Analytical strategies for the use of DNA probes," pages 1-25, see page 19.	11